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Substrate selection in ubiquitination reactions is achieved by ubiquitin ligases, which simultaneously bind both the target protein and a ubiquitin conjugating enzyme. While recent data indicates that there are a large number of distinct ubiquitin ligases that are responsible for the destruction of hundreds or perhaps thousands of proteins, there are currently no general methods to identify proteins whose levels are controlled by this mechanism. The development of general methods to systematically identify proteins whose abundance is regulated has major implications for: (1) elucidating proteolytic components of signaling pathways such as those activated in response to oncogenes and growth inhibitory factors, (2) identifying candidate drug targets whose altered destruction leads to therapeutic benefit, and (3) identifying genes that are required for the proper destruction of particular proteins of interest. We propose to merge our expertise in gene discovery approaches in yeast and mammalian cells with our experience in the molecular biology of regulated proteolysis to devise techniques that will facilitate the systematic identification of proteins derived from breast cell cDNA libraries whose levels are regulated.

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FOREWORD

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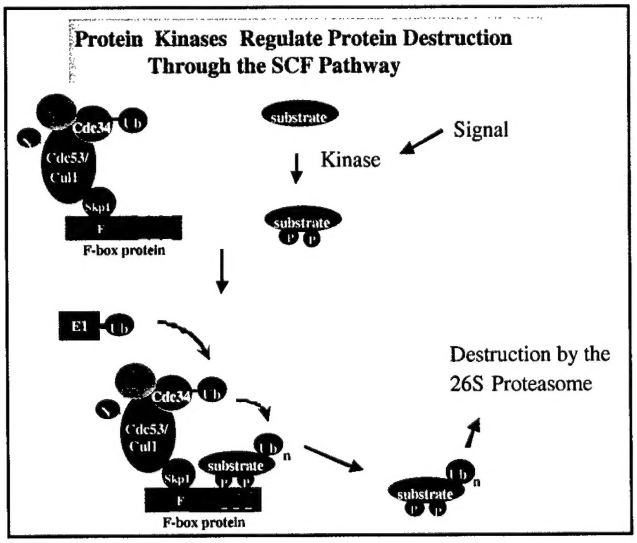
Introduction

Protein ubiquitination requires three components: E1, E2, and E3 (Hershko and Ciechanover, 1998). In the first step, a ubiquitin-activating enzyme (E1) is charged with ubiquitin through a thiol-ester linkage. This ubiquitin is then transferred to one of a dozen or so ubiquitin conjugating enzymes (E2) also as a thiol-ester. The ubiquitin is finally transferred from the E2 to one or more lysine residues in the substrate with the aid of an E3 ubiquitin ligase. In essence, E3s function as substrate-specific adaptors by simultaneously binding substrate and the E2, although in some cases, E3s may also serve as an intermediate in the ubiquitin transfer process.

Much of our knowledge of E3s has come from genetic dissection of signaling pathways that involve one or more ubiquitin-dependent events (reviewed in Koepp et al., 1999; Patton et al., 1998). These studies have revealed 3 broad classes of E3s that are likely to be responsible for targeting the ubiquitination of hundreds of proteins: 1) the HECT domain class which includes E6-AP, 2) the ring finger class which include Cbl and MDM2, and 3) the cullin-based ubiquitin ligases which include SCF, VBC, and APC complexes. Given the size of these different protein families, it is clear that many aspects of the biology of these E3s are unexplored. There are at least 40 HECT domain proteins in the human genome and more than 250 ring-finger containing proteins that may be involved in ubiqutination.

The complexity of these systems is perhaps best exemplified by the cullin-based ligases of which the SCF complex is the best understood. These are multicomponent E3s that dock substrates with a core ubiquitin conjugating system via modular substrate specific adaptors (reviewed in Koepp et al., 1999). The core components include a member of the cullin family of proteins, which contains 6 members in mammals, a RING finger protein typified by Rbx1 and APC11, and an E2 (Cdc34 or Ubc4). These core complexes interact with distinct families of substrate specific adaptors to generate a large number of ubiquitin ligases with distinct functions. Our work has focused on the SCF sub-family of cullin-based ubiquitin ligases, which refers to the three major components (Skp1, Cul1, and a member of the F-box family of proteins) (See scheme on left). Through genetic and biochemical studies in budding yeast, we identified Skp1 as a component of the SCF that links Cul1 to F-box proteins (Bai et al., 1996; Skowyra et al., 1997). We also discovered the F-box motif as a Skp1 binding element that is found in a large number of proteins that can bind to particular ubiquitination substrates in a phosphorylation dependent manner (Bai et al., 1996; Skowyra et al., 1997). The timing of ubiquitination and destruction of many proteins are controlled by protein phosphorylation, including



cyclin-dependent kinase inhibitors such as p27 and Sic1, and G1 cyclins (cyclin E and Cln proteins). Through biochemical reconstruction of the SCF mediated ubiquitination of Sic1 and Cln1, we were able to demonstrate that distinct F-box proteins recognize distinct targets in a phosphorylation dependent manner and allow ubiquitination via an Rbx1/Cul1 dependent pathway (Skowyra et al., 1997, 1999; Kamura et al., 1999). In addition to the F-box motif that mediates interaction with Skp1, F-box proteins frequently contain C-terminal protein-protein interaction domains (Bai et al., 1996). The most common are WD40 and leucine-rich repeat domains. In an effort to understand the complexity of mammalian F-box proteins, we have isolated a large number of cDNAs encoding F-box proteins (Winston et al., 1999a). In total, >68 distinct mammalian F-box proteins are now known to exist. We have shown that one of these, β-TRCP, is responsible for the ubiquitination of IkB, an inhibitor of the NFkB transcription factor complex required for the cytokine response as well as β-catenin, a transcription factor that functions as an oncogene when not properly destroyed by ubiquitin-mediated proteolysis (Winston et al., 1999b). The Cul2-based ubiquitin ligase has more than 20 known substrate adaptor proteins called SOCS-box proteins (Hilton et al., 1998), one of which is the von Hippel-Lindau tumor suppressor protein (Lisztwan et al., 1999). Although the functions of the vast majority of F-box and SOCS-box proteins are unknown, the finding that the limited number of F-box proteins that have been characterized all function to ubiquitinate multiple target proteins suggests that this family of E3s will be responsible for ubiquitination of possibly hundreds of proteins.

The challenge in the post-genome era will be: 1) to identify proteins whose abundance is regulated, 2) to determine what ubiquitin ligase pathways contribute to destruction of specific targets, and 3) to determine how the activities of particular ligases are controlled. Historically, the identification of ubiquitinated proteins has occurred on a case by case basis, and as such, we have a very limited view of the number and types of proteins in the cell that are controlled by this pathway. Moreover, we have little information that addresses how particular oncogenic events affect either the activities of different classes of ubiquitin ligases or the access of such ligases to their substrates. This is due, in part, to the fact that generally applicable methods are not available for identifying proteins that are destroyed in response to particular stimuli or in particular cellular contexts. In rare cases, it has been possible to use genetic screens in yeast to identify targets of ubiquitin ligases for which mutants were available. However, this approach is generally limited to yeast and even in cases where particular mutants in E3 components are available, substrates have been difficult to identify. In addition, approaches such as two-hybrid systems have not been particularly useful in identifying targets of ubiquitin ligase components such as F-box proteins. Given the large number of substrate adaptors that we and others have identified and that are likely to be identified in the future as a result of genome sequencing efforts, the identification of their important substrates will continue to be a major challenge.

We are attempting to employ multiple proteins to the identification of proteins that are ubiquitination substrates. Initially we proposed two systems, one involving yeast as a model system, and one involving mammalian cells. We are continuing to work on these systems but in the mean-time, we have also initiated two other approaches that are beginning to yield results.

In this proposal, we seek to develop new technologies that will facilitate the identification of proteins normally expressed in breast epithelial cells whose levels are controlled by proteolysis. These efforts are complementary to ongoing efforts to dissect ubiquitination-dependent pathways and represent an initial step towards the goal of unraveling proteolysis pathways at the whole genome level. In addition to the initial goal of defining ubiquitination substrates, the proposed system will find widespread application to identify targets of specific ubiquitin ligases as well as proteins whose abundance is regulated in response to certain stimuli frequently associated with breast cancer and the transformed phenotype. For example, it is well known that oncogenes such as c-myc lead to rapid destruction of p27, promoting proliferation, and this is the type of regulation that our approach would uncover. The development of such a system would have a major impact, not only in our basic understanding of breast cancer processes, but also in the areas of gene discovery and molecular medicine through the identification of new ubiquitination targets that might serve as drug targets.

Two complementary approaches will be undertaken. One approach will take advantage of the facile genetics in budding yeast to identify targets of known ubiquitin ligase. Yeast has provided many of the important insights into ubiquitination pathways that have been shown to be general to all eukaryotes. GFP-tagged cDNAs under control of the ADH promoter will be integrated into the yeast genome and cells sorted into pools based on GFP levels. Particular ubiquitin ligases will then be introduced and cells then sorted for reduced GFP-fusion protein levels. In principle, this approach could be used for mammalian genes as well as for yeast genes. For example, we have shown that human SCF components function in yeast, so in principle a novel mammalian F-box protein could be introduced into yeast strains carrying GFP-tagged cDNAs corresponding to mRNAs found in mammary epithelial cells. Yeast cells expressing reduced levels of GFP, identified by cell sorting, will contain a candidate substrate for the ubiquitin ligase under examination. Alternatively, it may be possible to look for cells that increase GFP signals in response to mutation of a particular ligase in yeast. In a second approach, we will generate libraries of breast epithelial cells expressing GFP-tagged cDNAs via integrating retroviral vectors. The GFP-tagged cDNA will be linked via an IRES to a GFP variant such that sub-libraries of cells can be purified by sorting cells with a particular GFP/variant GFP ratio. Treatment of these sub-libraries with various stimuli that normally activate destruction of a particular protein will result in decreased levels of particular GFP-cDNA fusion proteins found in rare cells but will not alter the levels of the internal GFP variant control protein and these cells can be sorted from those cells where the GFP/GFP variant ratio is unchanged. Recovered cDNAs will encode genes whose destruction occurs as a consequence of the applied stimuli and constitute potential regulatory components of the process.

Body

Development of a library-based method for identification of ubiquitination substrates in yeast

Aim 1 of this proposal ultimately seeks to develop a yeast-based system that allows the identification of mammalian proteins that are targeted by particular ubiquitin ligases.

However, prior to developing the heterologous system, the approach will be validated using yeast genes as targets. The method we are developing involves the generation of large fusion libraries in which all yeast genes are fused to the GFP gene under the control of a constitutive ADH promoter. The initial goal of generating GFP-fusion libraries has been accomplished. The method we used is based on the universal plasmid subcloning (UPS) system (Liu et al., 1998). UPS was developed based upon the Cre-*loxP* site-specific recombination system of bacteriophage P1. *LoxP* is a 34 bp DNA sequence that consists of two 13 bp inverted repeats flanking an 8 bp central sequence. Cre is the site-specific recombinase that catalyzes recombination between two *loxP* sites. The Cre-*loxP*-mediated site-specific recombination catalyzes plasmid fusion between the univector (pUNI) containing the gene of interest and the recipient vector (pHOST) that carries the regulatory information. pUNI has a Km resistance gene and a conditional origin that only replicates in special bacteria. Cre-catalyzed fusion events between pUNI and a pHOST vector can be genetically selected by selecting for Km resistance in a strain that cannot repicate pUNI. This generates a recombinant plasmid in which the gene in pUNI is placed under the control of novel regulatory elements or fused in frame to a gene encoding a protein expressed from the host plasmid. UPS is also very efficient because up to 20% of pUNI and pHOST plasmids can be fused in a single reaction to generate the desired recombinant plasmids and this makes possible the transfer of whole libraries from one vector to another. Once a gene is present in the univector, whether placed there initially or because it was identified in a library screen as proposed here, the majority of subsequent cloning events for the same gene can be accomplished uniformly and systematically simply by fusing with different recipient vectors.

We constructed two libraries (a random sheared genomic library and a cDNA library) in pUNI50 (a univector derivative). In addition, we prepared a pHOST vector with the following properties: 1) A LEU2 selectable marker for selection in yeast. 2) A GFP gene under control of the constitutive ADH promoter. This GFP gene will lack a stop codon and will instead have a lox site at its C-terminus. The lox site has a single open reading frame that will be in frame with the coding sequence of GFP. 3) A suppressor tRNA gene SUP11 that is toxic on plasmids unless integrated into the chromosome. 4) A pBR322 origin of replication and an Ap resistance marker for replication and selection in bacteria. 5) A sfil restriction site allowing integration into the yeast genome. This base vector is referred to as pGFPlox. The random sheared yeast genomic library with 3 x 10e7 recombinants. This represents a break at every nucleotide in the yeast genome on average, so all 6200 yeast genes will be represented many times over in this library. The genomic DNA in this library is inserted directly next to the lox site on pUNI50. The yeast genomic library was introduced into pGFPlox by cre-mediated plasmid fusion and plasmid fusion events were selected by selecting for Ampicillin/Kanamycin resistance upon transformation into a bacterial strain that cannot propagate pUNI50. This library constitutes the first reporter library. A second plasmid fusion reaction is in progress to generate a cDNA fusion library. In preliminary studies, we have found that the copy number of plasmids containing centromeric sequences can vary by two-fold. This makes the use of fax sorting difficult. Therefore, we have engineered the SfiI site as indicating above into the plasmid so that we can integrate the plasmid in a single copy into the yeast genome. This should provide a more stable background upon which to perform experiments. We are currently generating an integrant library and we are also trying to

optimize flow cytometry conditions. Once this is accomplished, we will begin screening using yeast cells that are mutant for the F-box protein Cdc4.

Use of yeast as a model system to identify substrates of F-box proteins

Budding yeast contains 16 F-box proteins, several of which display homology with mammalian genes. To explore the use of yeast to find relevant F-box proteins for unstable proteins of interest, we generated a series of yeast strains in which various F-box proteins were deleted. The majority of yeast F-box proteins can be deleted without loss of viability. The exceptions are YOR080 of YJL240. Deletion of these F-box proteins leads to a cold sensitive phenotype. We also have in hand, temperature sensitive (ts) mutants in Cdc4, Met30, and Grr1; the three major classes of conserved F-box proteins.

We expressed human cyclin E (a gene that is frequently induced in breast cancer and is thought to represent an independent prognostic marker) in each of these strains, including those lacking non-essential F-box proteins. We found that cyclin E is unstable in the majority of these strains but is stabilized in cdc4 ts mutants and in YOR080 deletion mutants at the non-permissive temperature. Importantly, we demonstrated that SCF complexes composed of CDC4 or YOR080 were capable of ubiquitinating cyclin E in vitro. Consistent with this, we also found that cyclin E was stabilized in skp1, cdc53, and cdc34 ts mutants. To identify mammalian F-box proteins, we surveyed a collection of F-box proteins that we had isolated for those that would bind to cyclin E. We tested 18 such genes and found one, Fbw7, that interacts with cyclin E. We found that it interacts in a phosphorylation specific manner and could interact with a specific cyclin E-derived phosphopeptide that is important for cyclin E turnover in vivo. The use of such phosphopeptides offers a new approach to the identification of ubiquitination substrates and is discussed in the conclusions section as a possible new approach. We went on to demonstrate that SCFFbw7 complexes can catalyze cyclin E ubiqutination in vitro. Moreover, we used siRNA in mammalian cells and RNAi in Drosophila cells to demonstrate that Fbw7 and its Drosophila homolog are involved in cyclin E turnover. This work was published in **Science** and the manuscript is attached. As such, I have only briefly described the results of this work. In collaboration with Khandan Keyomarsi at MD Anderson Cancer Center, we found that a number of breast cancer cell lines lack Fbw7 expression whereas normal counterparts express the protein properly. We sequenced all 11 exons from 8 such cell lines but we were unable to find mutations in the coding region. One possibility is that the Fbw7 promoter is methylated in cancer cells and is therefore unable to function properly. Ongoing studies are testing whether Fbw7 is a tumor suppressor through generation and analysis of mice lacking the Fbw7 gene.

Development of retroviral systems for identification of ubiquitination substrates in mammalian cells

The goal of aim 2 is to develop a retroviral based system for identifying unstable proteins in mammalian cells. The basis of this is a dual-color flow cytometric assay. We have generated a retroviral vector in which YFP is followed by a lox site, an RS recombination site, and IRES and CYP. When combined with a Univector-based mammalian cDNA library, one

can obtain a YFP fusion library. Unwanted Univector DNA sequences can then be removed by the RS recombinase, which deletes vector sequences while retaining cDNA sequences in the retroviral vector. These cDNAs are then followed by IRES-CFP. We have generated a mammalian cDNA library in pUNI50, which contains the necessary RS sequences. During the coming year, we expect to continue to develop this system, as described in the proposal.

Research Accomplishments:

Year 1

*Development of a yeast system for identification of proteins whose stability is regulated by a specific ubiquitin ligase

*The use of a related system to identify F-box proteins involved in cyclin E turnover

*Demonstration that Fbw7 in mammalian cells controls cyclin E levels

Reportable outcomes.

Publications supported by this grant:

Koepp, D., Schaffer, L., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. (2001) Phosphorylation-dependent ubiquitination of cyclin E by a conserved SCF^{Fbw7} ubiquitin ligase. **Science** 294, 173-177.

Conclusion

There is a clear need to develop methods that allow ubiquitination substrates to be identified. We are employing yeast as a model system to identify ubiquitin ligases for specific proteins (such as cyclin E) and are attempting to develop general library-based approaches to identify proteins whose levels are controlled by a particular ubiquitin ligase. Through our analysis of cyclin E ubiquitination, we have found that small synthetic peptides containing motifs known to be involved in cyclin E turnover are capable of binding relevant F-box proteins. This suggests a general approach to the identification of F-box substrates in which peptide libraries are used to determine which F-box proteins bind to which sequences. We are currently expanding our effort to develop these approaches further. We have recently acquired a peptide synthesis robot that allows the synthesis of hundreds of different peptides on a membrane, which can then be used to test for binding of particular proteins. We are currently working to express various F-box proteins and test these for binding to degenerate phosphopeptide libraries. If this is successful, we should be able to identify consensus sequences for particular F-box proteins and then us database searches to identify human proteins that contain these sequences. These can then be used to test whether they are substrates.

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Appendix:

Award Number: DAMD17-01-1-0135

TITLE: Identification of Ubiquitination Substrates

PRINCIPAL INVESTIGATOR: Jeffrey Harper, Ph.D.

Published manuscript

Koepp, D., Schaffer, L., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. (2001) Phosphorylation-dependent ubiquitination of cyclin E by a conserved SCF^{Fbw7} ubiquitin ligase. **Science** 294, 173-177.

quencing reactions with purified PCR products were performed by using Big Dye Terminator chemistry and forward or reverse primers in separate sequencing reactions (Applied Biosystems, Foster City, CA). Reactions were analyzed by using a 3700 Sequence Analyzer (Applied Biosystems). Sequence traces were automatically analyzed by using PhredPhrap and Polyphred (*47*, *48*). For SNPs identified through this analysis, PCR Invader assays (Third Wave Technologies, Madison, WI) were designed and tested on 90 samples from the Polymorphism Discovery Resource panel (PDR90) (*49*). Successful assays were subsequently used to analyze samples from our study. Genotypes were assigned automatically by cluster analysis (M. Olivier *et al.*, in preparation). Differences among genotypes were analyzed by one-way ANOVA using STATVIEW 4.1 software (Abacus Concepts, Inc., Berkeley, CA). SNPs 1 to 4 are available in dbSNP under accession numbers ss3199913, ss3199914, ss3199915, and ss3199916, respectively.

35. Subjects were a combined subset of 501 healthy, nonsmoking Caucasian individuals aged >20 years (429 men, 72 women) who had participated in previous dietary intervention protocols (*50*, *51*) (R. M. Krauss *et al.*, unpublished data). All subjects had been free of chronic disease during the previous 5 years and were not taking medication likely to interfere with lipid metabolism. In addition, they were required to have plasma total cholesterol concentrations <6.74 mmol/liter (260 mg/dl), triacylglycerol <5.65 mmol/l (500 mg/dl), resting blood pressure <160/105 mm Hg, and body weight <130% of ideal. Each participant signed a consent form approved by the Committee for the Protection of Human Subjects at E. O. Lawrence Berkeley National Laboratory, University of California, Berkeley, and participated in a medical interview. Fasting blood samples were obtained from subjects eating their usual diets, and after 4 to 6 weeks of consuming diets containing high fat (35 to 46% energy) and low fat (20 to 24% energy) (*50*, *51*). Plasma lipid and lipoprotein measurements were performed as previously described (*50*, *51*). In addition, on the high- and low-fat diets, total lipoprotein mass was measured by analytic ultracentrifugation (*50*, *51*).
36. Of the 501 individuals in the original study, 388 were successfully genotyped by PCR amplification for the Sst I polymorphism as previously described (*16*, *28*).
37. To genotype the C/T SNP3 polymorphisms upstream of *APOAV*, oligonucleotides AV6-F-5′-GATTGATTCAAGATGCATTTAGGAC-3′ and AV6-R-5′-CCCCAGGAACTGGAGCGAAATT were used to amplify a 187-bp fragment from genomic DNA. The penultimate base in AV6-R was changed to T to create a Mse I site (TTAA) in the common allele. The PCR reactions were performed in 20 μl volumes containing 50 mmol/liter KCl, 10 mmol/liter tris (pH 8.3), 1.5 mmol/liter $MgCl_2$, 0.2 mmol/liter of each dNTP, 1 U of *Taq* DNA polymerase, and 200 pmol/liter of each primer. DNA was amplified under the following conditions: initial denaturation of 96°C for 2 min, followed by 32 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and a final step at 72°C for 3 min. PCR product (20 μl) was digested with 10 U of Mse I (New England Biolabs) at 37°C for 3 hours. The PCR products were size-fractionated on 3% agarose gels, stained with ethidium bromide, and visualized on an ultraviolet transilluminator.
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52. Animals were killed, and tissues were harvested for either total RNA isolation by using the RNAeasy-midi protocol (Qiagen) or for poly(A)$^+$ mRNA isolation by using the FastTrack 2.0 system (Invitrogen, Carlsbad, CA). About 10 μg of total RNA or 2 μg of poly(A)$^+$ mRNA were separated in 1.0% agarose by gel electrophoresis and the RNA was transferred to a charged nylon membrane (Ambion, Austin, TX). The RNA blots were hybridized with [α-^{32}P]dCTP random-primed *apoAV* probes in ULTRAhyb buffer (Ambion). Probe templates were generated by PCR amplification of liver cDNA with degenerate primers degApoAV-F2-5′-GCGCGTGGTGGGRGAAGACA-3′ and degApoAV-R2-TCGCGCAGCTGGTCCAGGTT-3′. Filters were washed in 2× saline sodium citrate at room temperature for 20 min and in 0.1× SSC at 42°C for 20 min, followed by autoradiography visualization.
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Phosphorylation-Dependent Ubiquitination of Cyclin E by the SCF^{Fbw7} Ubiquitin Ligase

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Cyclin E binds and activates the cyclin-dependent kinase Cdk2 and catalyzes the transition from the G_1 phase to the S phase of the cell cycle. The amount of cyclin E protein present in the cell is tightly controlled by ubiquitin-mediated proteolysis. Here we identify the ubiquitin ligase responsible for cyclin E ubiquitination as SCF^{Fbw7} and demonstrate that it is functionally conserved in yeast, flies, and mammals. Fbw7 associates specifically with phosphorylated cyclin E, and SCF^{Fbw7} catalyzes cyclin E ubiquitination in vitro. Depletion of Fbw7 leads to accumulation and stabilization of cyclin E in vivo in human and *Drosophila melanogaster* cells. Multiple F-box proteins contribute to cyclin E stability in yeast, suggesting an overlap in SCF E3 ligase specificity that allows combinatorial control of cyclin E degradation.

Passage through the cell cycle is controlled by the activity of cyclin-dependent kinases (CDKs) (*1*). Cyclin E is the regulatory subunit of Cdk2 and controls the G_1 to S phase transition, which is rate-limiting for proliferation. Cyclin E is tightly regulated by ubiquitin-mediated proteolysis, which requires phosphorylation on Thr^{380} and Cdk2 activation (*2*–*4*). Failure to properly regulate cyclin E accumulation can lead to accelerated S phase entry (*5*), genetic instability (*6*), and tumorigenesis (*7*). Elucidating the mechanism controlling cyclin E destruction has important implications for understanding control of cell proliferation during development and its subversion by tumorigenesis.

The formation of polyubiquitin-protein conjugates, which are recognized and destroyed by the 26*S* proteasome, involves three components that participate in a cascade of ubiquitin transfer reactions: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a specificity factor (E3) called a ubiquitin ligase (*8*). E3s control the specificity of target protein selection and therefore are key to controlling individual target protein abundance.

The SCF (Skp1/Cullin/F-box protein) comprises a large family of modular E3s that control ubiquitination of many substrates in a phosphorylation-dependent manner (*9*). SCF complexes contain four subunits: Skp1, Cul1 (Cdc53), Rbx1, and an F-box–containing pro-

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tein. F-box proteins, over 50 of which have been identified in mammals (*10*, *11*), bind Skp1 through the F-box motif (*12*) and mediate substrate specificity of SCF complexes by binding substrates through protein-protein interaction domains, often WD40 repeats or leucine-rich repeats (LRRs) (*13*, *14*).

Several observations suggest that accumulation of cyclin E might be controlled through the SCF pathway. Cyclin E, like many SCF substrates, requires phosphorylation for destruction, and mice lacking Cul1 accumulate cyclin E (*15*, *16*). Because Cul3 mutant mice also show increased amounts of cyclin E (*17*), it is not clear if the effects of either cullin are direct. Stability of cyclin E expressed in *Saccharomyces cerevisiae* depends on phosphorylation of Thr^{380}, suggesting a conserved mechanism in yeast and mammals (*3*). Therefore, we exploited the genetics of *S. cerevisiae* to explore the contribution of SCF to cyclin E ubiquitination. We used a stability assay to perform a pulse-chase analysis of cyclin E protein in wild-type and *skp1-11*, *cdc34-2*, or *cdc53-1* mutants. To prevent cell cycle position effects, we arrested cells in S phase by addition of 200 mM hydroxyurea throughout the experiment. Cyclin E was unstable in wild-type cells but stabilized in SCF mutant cells (Fig. 1A). We examined cyclin E stability in yeast F-box protein mutant strains *cdc4-1*, *grr1*, *ydr219*, *yjl149*, *yml088/ufo1*, *ynl230/ela1*, *ynl311*, and *yor080/dia2*. Cyclin E was stabilized in *cdc4-1* strains to an extent similar to that seen with core SCF mutants and was also stabilized in *yor080* mutants (Fig. 1A). Cdc4 and Yor080 contain WD40 and LRR motifs, respectively. We incubated recombinant SCF^{Cdc4} and SCF^{Yor080} complexes with recombinant cyclin E–Cdk2, E1, Cdc34 (E2), Ub, and adenosine triphosphate (ATP) (Fig. 1B). Ubiquitination of cyclin E was detected with both complexes in an F-box– and ubiquitin-dependent manner (Fig. 1B). Ubiquitination was also stimulated by phosphorylated cyclin E as it was largely prevented when catalytically inactive cyclin E–$Cdk2^{KD}$ complexes were used as substrate (Fig. 1B).

To find the mammalian F-box protein that recognizes cyclin E, we surveyed previously identified F-box proteins (*11*) for those that bound cyclin E either after coexpression in insect cells or in vitro using ^{35}S-methionine–labeled translation products and immobilized glutathione *S*-transferase (GST)–cyclin E–CDK2 complexes. Seventeen F-box proteins were tested, including 16 that contained either WD40 or LRR motifs (*18*). Of these, only the WD40-containing Fbw7 (*19*) bound specifically to GST–cyclin E–Cdk2 but not to GST alone (Fig. 1C) (*20*). This interaction was specific

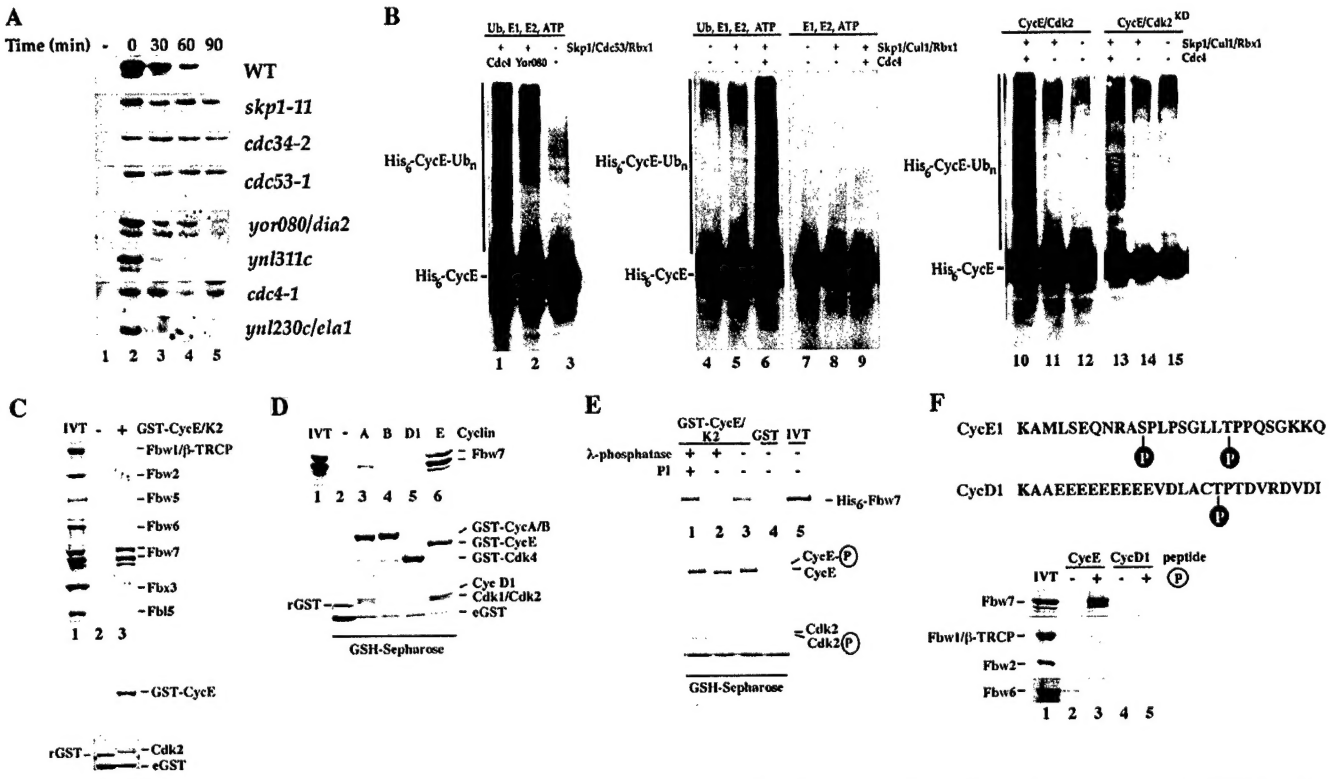

Fig. 1. Interaction between cyclin E and SCF components in yeast and mammalian cells. (**A**) Stabilization of cyclin E in *skp1-11*, *cdc34-2*, *cdc53-1*, *cdc4-1*, and *yor080* mutants (*12*, *30*). Strains of the indicated genotypes were grown in medium containing raffinose; cyclin E expression was induced for 1 hour by galactose addition and at time = 0 was repressed by addition of glucose. Cells were harvested at the indicated times, and the abundance of cyclin E was determined by immunoblotting. Extracts from uninduced cells are shown in lane 1. WT, wild type. (**B**) Cyclin E is ubiquitinated in vitro by SCF complexes. SCF^{Cdc4} or SCF^{Yor080} complexes were purified from insect cells (*13*) and supplemented with ubiquitin (Ub), E1, Cdc34 (E2), and ATP, as indicated, before addition of His_6–cyclin E–Cdk2 purified from insect cells (*13*). (**C**) GST–cyclin E–Cdk2 binds Fbw7. Immobilized GST–cyclin E–Cdk2 (lane 3) or GST (lane 2) was incubated with in vitro–translated F-box proteins (*31*, *32*). Lane 1 contains in vitro translation (IVT) product (33% of input). The bottom panel shows GST–cyclin E–Cdk2 and GST as detected by Coomassie staining. The positions of endogenous insect cell GST protein (eGST) and recombinant GST (rGST) are indicated. (**D**) Fbw7 preferentially binds cyclin E–Cdk2. The indicated Cdk complexes (lanes 2 to 6) were purified from insect cells and used for in vitro binding with Fbw7 as above. Cyclins were fused to GST for affinity purification, except for cyclin D1 where GST-Cdk4 is used. (**E**) Phosphorylation-dependent association of Fbw7 with cyclin E–Cdk2. Immobilized GST–cyclin E–Cdk2 was treated with λ-phosphatase in the presence (lane 1) or absence (lane 2) of phosphatase inhibitors (PI) before in vitro binding to His_6-Fbw7. Untreated GST–cyclin E–Cdk2 (lane 3) and GST (lane 4) were used as controls. Binding reactions were performed as in (C). (**F**) Immobilized cyclin E– or cyclin D–derived peptides with or without phosphorylation were incubated with Fbw7, Fbw1 (β-TRCP), Fbw2, and Fbw6 IVT products as in (C). The peptide sequence and sites of phosphorylation (P) are indicated (*33*).

for cyclin E as Fbw7 did not interact tightly with other cyclin-Cdk complexes (Fig. 1D). The interaction between Fbw7 and cyclin E was phosphorylation-dependent (Fig. 1E). Furthermore, Fbw7 bound specifically to a phosphopeptide containing the region of cyclin E required genetically for ubiquitination (Fig. 1F). Thus, the properties of Fbw7 are consistent with the predicted properties of a cyclin E ubiquitin ligase.

The mouse and human Fbw7 cDNA encodes a protein of 627 amino acids containing seven WD40 repeats (Fig. 2, A and B). The presence of stop codons in all three reading frames of the 5′ untranslated region (UTR) indicates that the encoded open reading frame (ORF) is full-length. Database searches revealed substantial sequence similarity with *Caenorhabditis elegans sel-10*, which is involved in the presenilin (*sel-12*) and Notch/lin-12 pathways (*21*), and the predicted protein encoded by *Drosophila melanogaster* CG15010 (*DmFbw7*). Among *S. cerevisiae* F-box proteins, Fbw7 is 28% identical to Cdc4 (Fig. 2A). The relationship between *sel-10* and a partial cDNA containing two COOH-terminal WD40 repeats from Fbw7 was noted previously (*21*). The extreme NH_2-terminus of Fbw7 contains a 23-residue stretch (residues 7 to 29) of highly hydrophobic amino acids recognized by the SMART protein analysis program as a transmembrane domain (*22*).

To examine the importance of the WD40 motifs in cyclin E recognition, we searched for basic residues located on the surface of the β-propeller structure that are conserved in Hs-Fbw7, Cdc4, Sel-10, and DmFbw7 but not in other Fbw proteins. Such residues would be candidates for phosphorylation-dependent interaction with ubiquitination targets. Arg^{417}, Arg^{457}, and Arg^{495}, located in WD40 repeats 3, 4, and 5, met these criteria (Fig. 2, A and D). These residues were independently replaced with alanine, and the resulting proteins were tested for binding to GST–cyclin E in vitro. Mutation of Arg^{417} or Arg^{457} abolished binding to cyclin E, whereas mutation of Arg^{495} reduced binding (Fig. 2C).

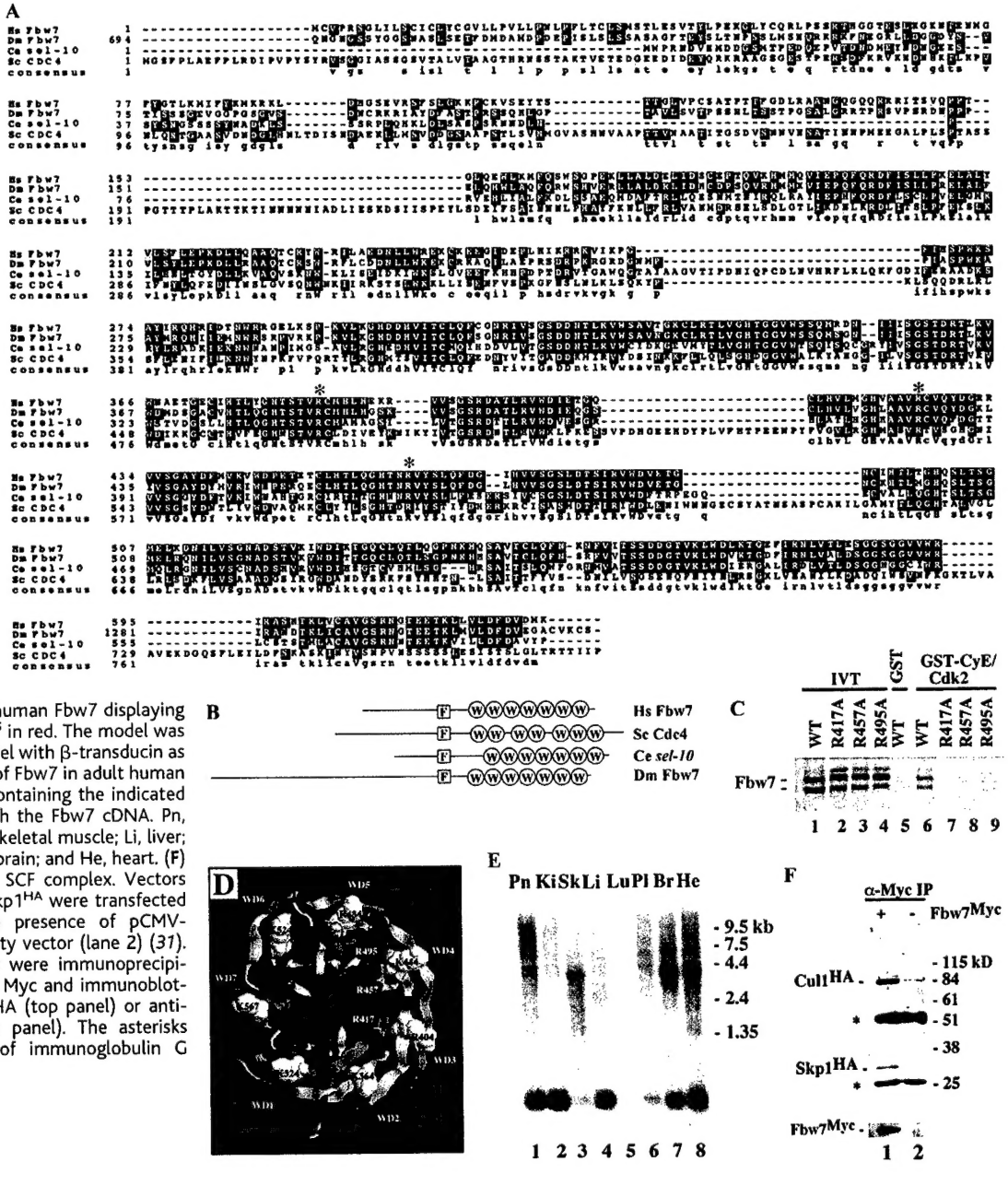

Fig. 2. Characterization of the WD40-repeat–containing F-box protein, Fbw7. **(A)** Conservation between human (Hs) Fbw7 and *C. elegans* (Ce) *sel-10*, *S. cerevisiae* (Sc) Cdc4, and *D. melanogaster* (Dm) Fbw7 (*33*). Identical residues are shaded black and similarities are shaded gray. Asterisks indicate conserved arginine residues required for cyclin E binding. **(B)** Domain structures of Fbw7 homologs. F, F-box; W, WD40 repeat. **(C)** Three surface arginines on Fbw7 are required for binding cyclin E. Wild-type (WT) and mutant Fbw7 IVT products were used for binding with GST–cyclin E–Cdk2 (lanes 6 to 9) or GST (lane 5). One-third of the input is shown (lanes 1 to 4). **(D)** Model of the β-propeller structure of human Fbw7 displaying Arg^{417}, Arg^{457}, and Arg^{495} in red. The model was generated with Swissmodel with β-transducin as template. **(E)** Expression of Fbw7 in adult human tissues. Northern blots containing the indicated mRNAs were probed with the Fbw7 cDNA. Pn, pancreas; Ki, kidney; Sk, skeletal muscle; Li, liver; Lu, lung; Pl, placenta; Br, brain; and He, heart. **(F)** Fbw7 assembles into an SCF complex. Vectors expressing $Cul1^{HA}$ and $Skp1^{HA}$ were transfected into 293T cells in the presence of $pCMV\text{-}Fbw7^{Myc}$ (lane 1) or empty vector (lane 2) (*31*). After 48 hours, extracts were immunoprecipitated with antibodies to Myc and immunoblotted with antibodies to HA (top panel) or antibodies to Myc (bottom panel). The asterisks indicate the positions of immunoglobulin G heavy and light chains.

Fbw7 mRNA is abundant in adult brain, heart, and skeletal muscle, tissues with a high percentage of terminally differentiated cells (Fig. 2E). Cotransfection of vectors encoding Myc-tagged Fbw7 with hemagglutinin (HA)-tagged Cul1 and HA-tagged Skp1 in 293T cells allowed detection of Fbw7 in SCF complexes, consistent with involvement of Fbw7 in ubiquitination (Fig. 2F).

We tested cyclin E ubiquitination in reticulocyte lysates in which either Fbw7 or Fbw2 had been translated. Ubiquitinated forms of cyclin E were observed in the presence of Fbw7 but not Fbw2 (Fig. 3A). Fbw7-dependent ubiquitination of cyclin E was also achieved in more purified systems. His_6-Fbw7 was affinity-purified on immobilized GST–cyclin E–Cdk2 (Fig. 3, B and C) or antibodies to His_6 (Fig. 3D) and used in ubiquitination reactions. Cyclin E ubiquitination was dependent on Fbw7 (Fig. 3, B and C) and was stimulated by Cul1-Rbx1 (Fig. 3, B to D). A small fraction of Fbw7 was associated with endogenous Cul1 in reticulocyte lysates (*20*). The pattern of conjugates was distinctly different when a form of ubiquitin that cannot undergo polyubiquitination (GST-Ub^{R-A}) was included in the reaction mixture (Fig. 3C), indicating that the larger forms of cyclin E are ubiquitin conjugates. The ubiquitination reaction was also stimulated by phosphorylation of cyclin E (Fig. 3D) and was reduced when the cyclin E Thr^{380} → Ala (T380A) mutant was used as substrate (Fig. 3E).

If Fbw7 is rate-limiting for controlling cyclin E abundance, overexpression of Fbw7 should lead to decreased amounts of cyclin E. To test this, we transfected 293T cells with vectors encoding cytomegalovirus (CMV) promoter–driven cyclin E, Cdk2, and either Fbw7 or empty vector and assayed cyclin E amounts by immunoblotting. Cells cotransfected with Fbw7 reproducibly had smaller amounts of cyclin E but constant amounts of Cdk2 (Fig. 4A).

Conversely, inhibition of Fbw7 should lead to increased accumulation of cyclin E. To test this, we used the small interfering RNA (siRNA) technique to reduce expression of Fbw7 in HeLa cells (*23*). Cells transfected with a double-stranded RNA (dsRNA) oligo corresponding to Fbw7 showed increased accumulation of cyclin E when compared with cells transfected with a control dsRNA oligo (Fig. 4B). Amounts of Cdk2 and bulk Cdk2 activity remained unaffected (Fig. 4B) (*20*). The amount of p27 was similar in both Fbw7- and green fluorescent protein (GFP)–inhibited cells at the 48-hour time point, indicating that the accumulation of cyclin E in Fbw7-inhibited cells was not substantially influenced by p27 (*20*). To assess the effect of Fbw7 on cyclin E stability, we used the siRNA-inhibited cells for a pulse-chase analysis of cyclin E (*2*). Cells were labeled in vivo with ^{35}S-methionine, samples were taken at the indicated times after replacement with medium containing unlabeled methionine, and cyclin E was immunoprecipitated (Fig. 4C). In the GFP siRNA cells, cyclin E was unstable, whereas in Fbw7-inhibited cells, cyclin E remains stable for the course of the experiment. Immunoblotting of the immunoprecipitates indicated that cyclin E amounts remained constant throughout the experiment.

We also used the RNA interference (RNAi) technique to ablate Fbw7 in *D. melanogaster* (S2) cells (*24*). Transfection of S2 cells with dsRNAs corresponding to various portions of the *DmFbw7* gene reduced amounts of DmFbw7 mRNA (Fig. 4D) and increased accumulation of cyclin E protein but not that of a control protein, Mle1 (Fig. 4D). In contrast, amounts of cyclin E mRNA were unaltered or slightly reduced, indicating that DmFbw7 regulates cyclin E through a posttranscriptional mechanism. Control dsRNAs had no effect on DmFbw7 or cyclin E (Fig. 4E). RNAi with the COOH-terminal fragment of Fbw7 was less efficient in destabilizing Fbw7 mRNA; thus, smaller increases in cyclin E accumulation were observed.

In this report, we show that SCF^{Fbw7}-related ligases control the stability of cyclin E in a manner conserved through evolution. The finding that different E3s can control cyclin E levels in yeast may have implications for control of cell proliferation in mammals. Such a role would allow multiple signals to be independently integrated through different E3s to control cyclin E levels and cell proliferation. This could allow tissues to exert combinatorial control of proliferation and differentiation, consistent with the tissue-specific expression of

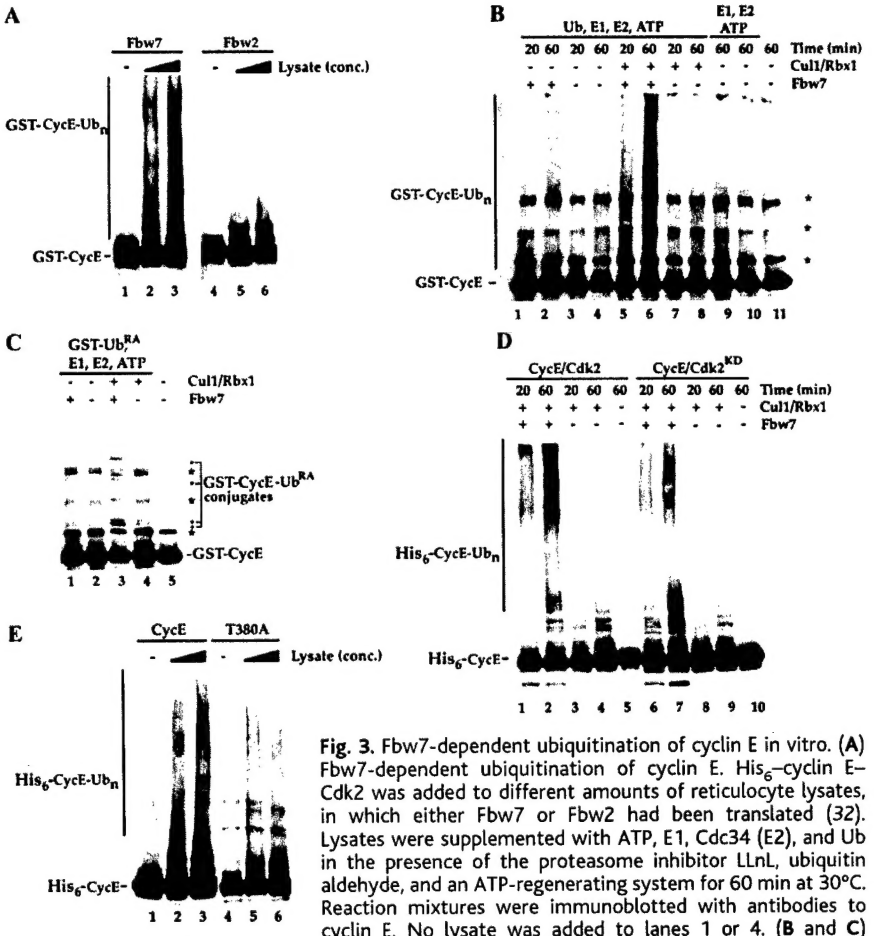

Fig. 3. Fbw7-dependent ubiquitination of cyclin E in vitro. **(A)** Fbw7-dependent ubiquitination of cyclin E. His_6–cyclin E–Cdk2 was added to different amounts of reticulocyte lysates, in which either Fbw7 or Fbw2 had been translated (*32*). Lysates were supplemented with ATP, E1, Cdc34 (E2), and Ub in the presence of the proteasome inhibitor LLnL, ubiquitin aldehyde, and an ATP-regenerating system for 60 min at 30°C. Reaction mixtures were immunoblotted with antibodies to cyclin E. No lysate was added to lanes 1 or 4. **(B** and **C)** Ubiquitination of GST–cyclin E by prebound His_6-Fbw7. (B) Immobilized GST–cyclin E–Cdk2 was incubated with reticulocyte extracts in the presence or absence of Fbw7. Beads were supplemented with E1, Cdc34 (E2), ATP, and either ubiquitin (Ub; 100 μg/ml) or GST-Ub^{RA} (100 μg/ml). Where indicated, 50 ng of a purified Cul1-Rbx1 complex was added. The asterisks indicate the positions of three proteins that cross react with the monoclonal antibodies to cyclin E. (C) As in (B), but GST-Ub^{RA} was used in place of ubiquitin. **(D)** Cyclin E phosphorylation enhances ubiquitination of cyclin E by SCF^{Fbw7}. Reticulocyte lysates with or without His_6-Fbw7 were immunoprecipitated with antibodies to His tag, supplemented with cyclin E–Cdk2 (or cyclin E–$Cdk2^{KD}$), E1, Cdc34 (E2), ubiquitin, and ATP and incubated at room temperature for the indicated time. Samples were treated as in (B). **(E)** Phophorylation of Thr^{380} enhances ubiquitination of cyclin E. Reactions were performed as in (A), but cyclin E T380A was also used as substrate.

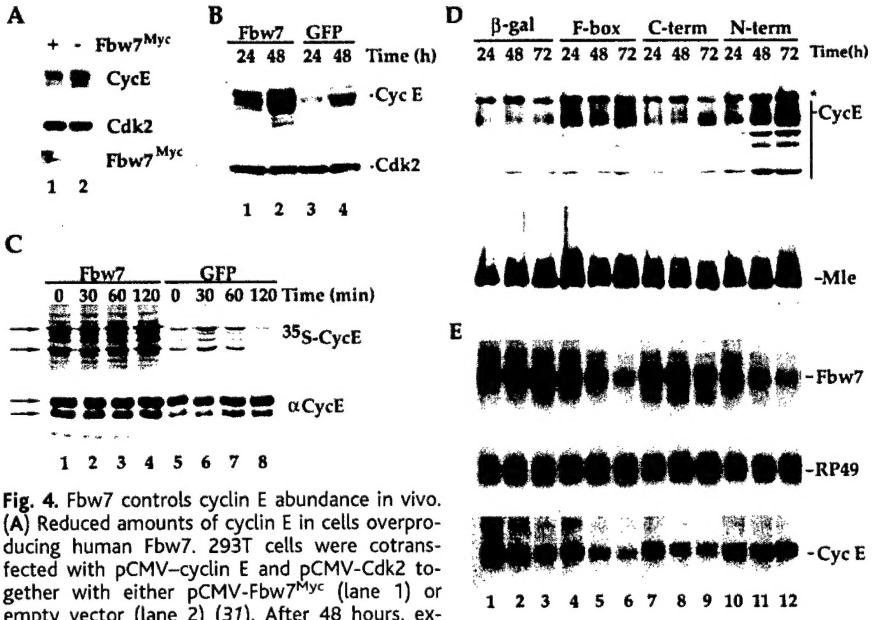

Fig. 4. Fbw7 controls cyclin E abundance in vivo. (**A**) Reduced amounts of cyclin E in cells overproducing human Fbw7. 293T cells were cotransfected with pCMV–cyclin E and pCMV-Cdk2 together with either pCMV-Fbw7^{Myc} (lane 1) or empty vector (lane 2) (*31*). After 48 hours, extracts were prepared and immunoblotted with antibodies to Cdk2, cyclin E, or Myc. (**B**) Accumulation of cyclin E in HeLa cells transfected with Fbw7 siRNA but not GFP siRNA. Cells were transfected as described (*23*, *34*). At the indicated times, cells were harvested and cell lysates were generated. Samples were immunoblotted with antibodies to cyclin E or Cdk2. (**C**) Cyclin E is stable in Fbw7-inhibited cells. Cells were transfected as in (B), and pulse-chase analysis was performed as described (*2*). Medium containing unlabeled methionine was added at time = 0. Samples were also immunoblotted with monoclonal antibodies to cyclin E (bottom panel). Arrows indicate the two major forms of cyclin E. (**D** and **E**) Accumulation of *DmCycE* in response to ablation of *DmFbw7* by RNA interference. S2 cells were transfected with dsRNA corresponding to the NH_2-terminal (N-term), COOH-terminal (C-term), or F-box region of *DmFbw7* or against β-galactosidase (β-gal) as a control (*34*). At the indicated times, cells were harvested and used to generate protein extracts and total RNA. (D) Cell extracts were immunoblotted with polyclonal antibodies against *DmCycE* or *maleless* (Mle). (E) Messenger RNA was subjected to Northern blotting with probes directed toward *DmFbw7*, *DmCycE*, or a ribosomal RNA (RP49).

Fbw7. Cells lacking the F-box protein Skp2 also accumulate cyclin E (*25*). However, this effect may be an indirect result of the accumulation of the Skp2 substrate, p27 (*26*, *27*). Individual E3s often control the ubiquitination of multiple substrates (*9*); therefore, controlling accumulation of cyclin E through expression of a particular E3 may limit the function of other signaling pathways as a consequence. Thus, using different E3s to control cyclin E might lead to regulation of different constellations of signaling pathways in a tissue-specific manner. It is likely that Fbw7 controls the ubiquitination of other proteins in addition to cyclin E. Putative substrates include Notch and Presenilin proteins, as the *C. elegans* homolog *sel-10* has been implicated in the control of both Notch and Presenilin signaling (*21*, *28*).

As a negative regulator of cyclin E, Fbw7 is a potential tumor suppressor. Consistent with this, we have observed that amounts of Fbw7 mRNA are decreased in breast tumor lines that have increased amounts of cyclin E (see supplemental Web figure 1 on *Science* Online at www.sciencemag.org/cgi/content/full/294/5540/173/DC1). Thus far, we have not identified mutations in the Fbw7 gene in these or other tumors. However, Fbw7 maps to 4q32, a site of loss of heterozygosity in a number of cancers (*29*). Additional studies will be required to resolve Fbw7's role in tumorigenesis.

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18. F-box proteins tested for cyclin E binding include Fbw1 (β-TRCP), Fbw2 (MD6), Fbw5, Fbw6, Fbw7, Fbl1 (Skp2), Fbl2, Fbl3a, Fbl4, Fbl5, Fbl6, Fbl7, Fbl8, Fbl11, Fbl12, and Fbx3. The Fbw6 construct for in vitro translation was generated from an expressed sequence tag (EST) previously named Fbx29 (GenBank accession number AF176707) (*11*).
19. We previously identified the F-box motif in EST clone AI836688 as Fbx30 (*11*). Further sequence analysis of a longer cDNA (GenBank accession number AY033553) revealed WD40 repeats, and it was renamed Fbw7 according to convention (*10*, *11*).
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30. Description of the yeast F-box protein mutants will be presented elsewhere. The Fbw7 ORF lacking the stop codon was amplified by polymerase chain reaction from the EST 3347354 and inserted into pCR2.1 and pcDNA3.1 Myc-His. The His_6-Fbw7 vector was generated similarly except that the amplified ORF contained a stop codon.
31. 293T cells were transfected with Lipofectamine (Invitrogen). For association of cyclin E–Cdk2 and other cyclins with F-box proteins in vitro, immobilized GST-cyclin-Cdk or GST was incubated (1 hour, 4°C) with ^{35}S-methionine–labeled in vitro–translated F-box proteins and washed four times before electrophoresis. In some experiments, GST–cyclin E–Cdk2 was treated with 400 units of λ-phosphatase for 60 min at 30°C and then washed twice before binding.
32. SCF complexes were assembled by coexpression of Flag-Skp1, Cdc53, Rbx1, and either Cdc4 or Yor080 in insect cells and used as described (*13*). Some SCF^{Cdc4} complexes were assembled on GSH-Sepharose (Amersham Pharmacia) with human GST-Cul, Skp1, and Rbx1. His_6-tagged E1 enzyme and Cdc34-E2 were purified from yeast and bacteria, respectively. For ubiquitination in crude extracts, reticulocyte lysates programmed with either Fbw7 or Fbw2 were supplemented with an ATP regeneration system, E1 (100 ng), Cdc34 (E2) (300 ng), ubiquitin (5 μg), and His_6-cyclin E-Cdk2. For ubiquitination of GST–cyclin E prebound to SCF^{Fbw7}, GST–cyclin E–Cdk2 was incubated with reticulocyte lysate programmed with Fbw7 or lysate lacking Fbw7 at 4°C (60 min). Washed complexes were supplemented as described above. To examine ubiquitination by immune complexes containing His_6-Fbw7, we supplemented in vitro–translated His_6-Fbw7 immobilized on antibody to His tag beads as described above.
33. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
34. RNA interference was performed as described (*24*) except that Effectene (Qiagen) was used for transfection. dsRNAs corresponded to nucleotides 1 to 505 (NH_2-terminal), 2678 to 3159 (F-box), and 3469 to 3981 (COOH-terminal) of the Fbw7 coding region. The siRNA oligo corresponded to nucleotides 713 to 735 of the human Fbw7 coding region.

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